Signal Transduction During Cardiac Hypertrophy: New Insights

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Introduction

In the past decade it has become apparent that many diseases result from aberrations in cell signalling pathways. Because of their link to disease, all major pharmaceutical and biotechnology companies have active drug discovery programmes based on understanding signal transduction pathways. The so-called signal transduction therapy is gaining importance as intervention of signalling processes is considered a novel approach to disease management through the development of therapeutic agents to control specific cellular responses. The first step in this process is the identification of appropriate signalling targets. This review summarises the recent work and the role of two such elementary cellular targets viz. calcium messenger system and protein kinase C (PKC) in cardiac hypertrophy.

Cardiovascular diseases (CVDs) are at present the leading cause of death worldwide. There is an international concern that CVDs will overtake lower respiratory infections to become the Number One killer in developing countries by the year 2020. Among the cardiovascular diseases, heart failure is the major cause of disability and morbidity worldwide and in the United States alone it affects about 700,000 individuals each year1. Heart disease can arise from extrinsic stimuli such as hypertension or from intrinsic defects within the heart itself. Hypertrophic cardiomyopathy (HCM) is the most common form of intrinsic heart disease and has been cited as the most frequent cause of sudden death in young people1.

Cardiac Hypertrophy

Hypertrophy, a generalised enlargement of the myocardium (an increase in cell size without cell division), is a fundamental response of cardiac myocytes to common clinical disorders such as hypertension, valvular heart disease, myocardial infarction and congenital heart disease. It is the primary mechanism by which adult cardiac myocytes adapt to an increasing workload because most of them lose their capacity to divide shortly after birth. Thus hypertrophy is initially a beneficial adaptive response to balance the increased stress on the myocardium. But excessive cellular hypertrophy caused by prolonged stress eventually progresses to heart dilatation, functional insufficiency and failure. In fact, virtually all the forms of myocardial dysfunction (even that caused by gradual cell loss during normal aging) are associated with various degrees of hypertrophy. Hence, there is increasing interest in hypertrophic signalling with the hope that targeting specific signalling molecules by drugs or even gene therapy might be useful in heart failure.

In vitro models of cardiac hypertrophy include cultures of neonatal cardiac myocytes that are treated with humoral factors (such as angiotensin II, norepinephrine or endothelin-1) or subject to direct mechanical stress through stretching2. A variety of stresses can cause hypertrophy ranging from a congenitally weak heart muscle to a narrowed aortic valve, high blood pressure, or death of parts of the muscle in a heart attack. Most of these causes seem to have one thing in common, viz. they raise calcium levels in heart cells. In other words, all hypertrophic stimuli increase intracellular calcium levels ([Ca\(^{2+}\)]) and activate multiple signal transduction pathways.

Multiple Regulators of [Ca\(^{2+}\)], in Cardiac Myocytes

Cardiac contraction and relaxation are mediated by the rapidly changing calcium concentrations around the myofibrils. The force of cardiac contraction is determined in part by the amount of Ca\(^{2+}\) that is released from the sarcoplasmic reticulum (SR) during a cardiac action potential. Ca\(^{2+}\) content of SR is in turn determined by the interplay between Ca\(^{2+}\) influx/efflux at the sarcolemmal membrane (plasma membrane), and Ca\(^{2+}\) accumulation/release at the SR membrane. Ca\(^{2+}\) influx at the sarcolemma occurs primarily through voltage-
gated Ca\textsuperscript{2+} channels, whereas the principal efflux mechanism is the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange system. Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange is a carrier-mediated process which couples the movement of three Na\textsuperscript{+} ions in exchange for a single Ca\textsuperscript{2+} ion moving in the opposite direction. Hence, it establishes a connection between the transmembrane gradient of Na\textsuperscript{+} and the [Ca\textsuperscript{2+}]. It is also a bidirectional transport process, capable of moving Ca\textsuperscript{2+} in either direction across the plasma membrane. However, because of the inwardly directed concentration gradient of Na\textsuperscript{+}, established by the action of the Na, K-ATPase, its principal mode of operation is in the direction of Ca\textsuperscript{2+} efflux. With each beat of the heart, the exchanger transports 25-30 percent of the Ca\textsuperscript{2+} released from the SR out of the cell\textsuperscript{1}. In the steady state, this Ca\textsuperscript{2+} efflux is balanced by an equivalent amount which enters the cell, primarily through Ca\textsuperscript{2+} channels. Changes in the driving force for Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange, therefore, exert a profound influence on the amount of Ca\textsuperscript{2+} taken up by the SR and on the force of contraction of the subsequent beats. Apart from Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, the major contribution to diastolic calcium lowering is made by the Ca\textsuperscript{2+}-ATPase of the SR (SERCA) that pumps cytosolic calcium into the lumen of SR. The SERCA pump is a 110 kD single peptide that moves two molecules of calcium from the cytoplasm of the myocyte into the sequestered space of SR per molecule of hydrolysed ATP. It is also a member of the P-type ion-motive ATPases that form a phosphoprotein intermediate during ion translocation. So far, five distinct SERCA isoforms that are encoded by three separate genes (SERCA 1-3) have been described in the literature\textsuperscript{4} and SERCA2 is mainly expressed in cardiac myocytes\textsuperscript{5}.

**Does Calcium Play a Role in Cardiac Hypertrophy?**

During recent years the role of Ca\textsuperscript{2+} in the development and maintenance of disease states has been demonstrated in a number of instances. Altered Ca\textsuperscript{2+} signalling has been reported in many cardiovascular diseases including heart failure. Since [Ca\textsuperscript{2+}]\textsubscript{i} levels regulate cardiac muscle contraction and relaxation, it is reasonable to suggest that elevated calcium has a role in the aetiology of cardiac hypertrophy and heart failure. In fact, abnormal transient fluxes in Ca\textsuperscript{2+} have been observed in various experimental models of cardiac hypertrophy as well as in explanted human hearts from patients with end-stage heart failure\textsuperscript{4}. Many studies indicate that myocyte hypertrophy is associated with the elevation of or enhanced sensitivity to intracellular calcium\textsuperscript{7}.

But this calcium overload hypothesis has been difficult to prove because abnormal alterations in transient Ca\textsuperscript{2+} fluxes may be secondary to structural and biochemical changes in Ca\textsuperscript{2+}-regulatory proteins associated with cardiac hypertrophy\textsuperscript{8}. Pathophysiological influences on SERCA2 gene expression have been demonstrated in animals undergoing pressure overload-induced cardiac hypertrophy\textsuperscript{9,10} and in human beings with end-stage heart failure\textsuperscript{11,12}. In each of these conditions, SERCA2 mRNA and protein levels are markedly decreased. Western blot analysis of myocardium from the failing human heart reveals decreased levels of SR-Ca\textsuperscript{2+} ATPase and increased sarcoluminal Na/Ca exchanger\textsuperscript{13}. More importantly, a recent study has demonstrated enhanced calcium transients, myocardial contractility and re-laxation in SERCA2 overexpressing transgenic mice\textsuperscript{14}. These results support the concept that reduced function of sarcoplasmic reticulum to accumulate calcium may reflect a major defect in excitation-contraction coupling in human heart failure. Enhancing the activity of SERCA2 in the failing heart leads to improved contractile performance and may, therefore, have further therapeutic implications.

Since cardiac myocytes are normally experiencing large fluctuations in [Ca\textsuperscript{2+}], with each contraction/relaxation cycle, many scientists doubt that [Ca\textsuperscript{2+}]\textsubscript{i} is the major trophic signal for hypertrophy. At the same time, it is intriguing to note that several intrinsic cardiomyopathies are caused by genetic mutations in contractile proteins that organise into repetitive units known as sarcomeres. Mutations have been identified in the genes\textsuperscript{15-18} encoding β-myosin heavy chain (MHC), cardiac troponin T, α-tropomyosin, myosin-binding protein C, myosin light chains (MLC) and cardiac α-actin. Genetic and functional data suggest that mutations which cause hypertrophic cardiomyopathy act as dominant negative alleles that impair crossbridge cycling and contractile function and interfere with sarcomere assembly\textsuperscript{17}. Normal sarcomeric function is associated with basal [Ca\textsuperscript{2+}]\textsubscript{i} levels that regulate contractility. It has been postulated that mutations in sarcomeric proteins lead to increases in [Ca\textsuperscript{2+}]\textsubscript{i} in order to maintain contractility and cardiac output\textsuperscript{19}. However, it is a paradox to observe that increases in basal [Ca\textsuperscript{2+}]\textsubscript{i} are also associated with cardiac hypertrophy. How calcium might affect hypertrophy was a long-standing question to many scientists.

**Signalling Pathway(s) in Cardiac Hypertrophy**

The missing link between calcium and hypertrophy was identified by Molkentin et al\textsuperscript{20} in a recent mouse
model study. They reported that the Ca²⁺-sensitive phosphatase calcineurin - the target of the immunosuppressive drugs cyclosporin A (CsA) and FK506 - is the critical mediator of cardiac hypertrophy and heart failure. While studying a protein called GATA4, a DNA-binding protein that turns on heart cell genes during hypertrophy, the team found that GATA4 binds to a protein called NFAT3 (NFAT stands for nuclear factor of activated T cells). The GATA proteins are a family of zinc finger proteins that are expressed early in cardiac development and may act separately from, or in concert with, the homeodomain proteins as crucial regulators of heart development. GATA4 contains the highly conserved DNA-binding domain that characterises this family of cell-type restricted transcriptional activators. The observation of binding of GATA4 to another transcriptional factor NFAT3 has excited many scientists and pointed out a novel signalling pathway as a target for heart failure therapies.

NFAT3 belongs to a family of proteins that regulate genes in response to [Ca²⁺], in activated T cells of the immune system. In unstimulated T cells, NFATs are localised in the cytoplasm but upon mitogenic stimulation (that accompanies increases in [Ca²⁺]), they become dephosphorylated by the Ca²⁺-dependent calcineurin and translocate to the nucleus where they activate the key T-cell growth factor interleukin-2. While basal [Ca²⁺], T-cells are finetuned by the concerted action Ca²⁺ pumps, exchangers and channels, a profound modulation of these Ca²⁺ transport proteins was also demonstrated during T-cell activation. A sustained increase in [Ca²⁺] levels and activation of calcineurin are critical to the immune response. Although calcineurin is present in most tissues, including the heart, its biological function in non-immune cells is not well understood. Similarly, NFAT also shows a wide-tissue distribution.

Molkentin et al. demonstrated that NFAT3, GATA4 and calcineurin synergistically activate marker genes for cardiac hypertrophy. This signal transduction pathway is very reminiscent of that active in T-cells, where calcineurin-dependent translocation of NFAT to the nucleus cooperates with AP1 to stimulate T-cell specific promoters. In T lymphocytes, a Ca²⁺-dependent calcineurin activates NFAT proteins by removing a phosphate group they carry. This allows NFAT to enter the nucleus and regulate the genes. Similar course of events is expected to happen when calcium levels rise in stressed hearts (Fig. 1). A central role for the calcineurin pathway in hypertrophy was suggested by blocking of the hypertrophic response in cultured myocytes (stimulated with angiotensin II or phenylephrine) by CsA or FK506. Additionally, cardiac-specific overexpression of constitutively active form of calcineurin caused a marked cardiac hypertrophy in transgenic mice, many of which progressed to congestive heart failure and sudden death (with similar physiological and pathological aspect of human heart failure). As expected, this cardiac hypertrophy was completely prevented by early treatment of the calcineurin transgenic animals with CsA or FK506, demonstrating that the phosphatase activity of
calcineurin is critical for the development of cardiac hypertrophy. Finally, the study also evidenced that transgenic mice overexpressing a constitutively active (nuclear localised, lacked N-terminal sequences) form of NFAT3 in the heart showed marked hypertrophy, whereas the wild-type protein had no effect. This clearly demonstrates that dephosphorylation of NFAT3 by calcineurin is the the rate limiting step in NFAT3-induced cardiac hypertrophy.

In another series of studies\textsuperscript{35}, three mouse cardiomyopathic models were produced with a common defect in contractility caused by perturbations in sarcomeric proteins. These mouse models exhibited HCM as a result of (i) cardiac-specific overexpression of actin-capping molecule tropomodulin, or (ii) lack of myosin light chain-2 protein, or (iii) overexpression of β-tropomyosin in the heart. CsA treatment of these transgenic mice prevented the development of dilated cardiomyopathy in every animal tested. In contrast to this, CsA treatment in transgenic mice overexpressing retinoid acid receptor in the heart (these animals develop HCM due to alterations of the expression of retinoid receptor-dependent genes) did not inhibit HCM. However, CsA treatment inhibited HCM in a rat model of pressure-overload hypertrophy (which resembles the extrinsic form of human heart disease). To confirm whether the effect of CsA or FK506 on cardiomyopathy was mediated through calcineurin, the scientists have also performed calcineurin enzymatic assays. Calcineurin activity was found to be doubled in tropomodulin overexpressing transgenic (TOT) mice, when compared to wild type hearts. As expected, TOT hearts treated with CsA showed a similar calcineurin activity as a wild type hearts. These data clearly confirm the activation of calcineurin in TOT mice HCM models. Because elevated [Ca\textsuperscript{2+}] levels are involved in the development of cardiomyopathy\textsuperscript{25,33}, these results strengthen the hypothesis that calcineurin mediates hypertrophic signalling in response to altered Ca\textsuperscript{2+} concentrations. Moreover, it is important to note that calcineurin is activated by prolonged increases in [Ca\textsuperscript{2+}], but not by transient Ca\textsuperscript{2+} spikes associated with the activation of Ca\textsuperscript{2+}/calmodulin-dependent kinase II (CaMKII) and the mitogen-activated protein kinase (MAPK)\textsuperscript{37,38}.

The message from the above studies is that calcineurin may be the nodal point in the conversion of elevated [Ca\textsuperscript{2+}] level ("life signal") to the trophic ("death") signal that sets cardiac hypertrophy in motion. Sustained increases in [Ca\textsuperscript{2+}] due to persistent hypertrophic stimuli and the associated altered expression of Ca\textsuperscript{2+}-regulatory proteins (pumps, channels and exchangers) could initiate a vicious cycle of events and result in heart failure. Atleast one way by which ubiquitous calcium signals could direct specific biological processes is through the tissue-specific expression of target molecules such as NFAT. As NFAT family members are expressed in different tissues\textsuperscript{39}, specificity of response to a Ca\textsuperscript{2+} stimulus could be refined by both different tissue distribution and DNA sequence recognition.

Additional Signalling Pathway

Though calcineurin is identified as a cellular target for a variety of Ca\textsuperscript{2+}-dependent signalling pathways culminating in cardiac hypertrophy, recent studies have also questioned the role of calcineurin at least in the haemodynamic load-induced hypertrophy. Calcineurin inhibitors (CsA and FK506) did not prevent the development of pressure-overload left ventricular hypertrophy (LVH) in two classic models (the spontaneously hypertensive rat and rats subjected to constricted abdominal aortas)\textsuperscript{30,31}. In another study\textsuperscript{32}, CsA was reported to attenuate pressure overload hypertrophy but at the same time, it has enhanced the susceptibility of the mice to decompensation and heart failure. These results demonstrate that pressure-overload hypertrophy can arise through calcineurin-independent pathways.

Similarly, it would be of importance to know whether Ca\textsuperscript{2+}/calcineurin signalling is involved in diabetes-induced cardiomyopathy. The existence of diabetes-induced cardiomyopathy in the absence of coexisting coronary atherosclerosis or systemic hypertension has been established in both humans and experimental animals\textsuperscript{33,34}. This condition is characterised by reductions in diastolic compliance, contractility, and the rate of myocardial relaxation. The mechanisms responsible for these diabetes-linked cardiac defects are not fully understood and may be quite different in the two diabetic states. A few studies\textsuperscript{35-37} have demonstrated that defective sarcolemma and sarcoplasmic reticular Ca\textsuperscript{2+} transport contribute to diabetic cardiomyopathy via altered cellular Ca\textsuperscript{2+} regulation. In the event of the alarming increases in diabetes-associated cardiovascular complications, future research should focus on unravelling the causal mechanisms of diabetes-induced cardiomyopathy.

PKC Isoforms in Cardiac Hypertrophy

Recent studies indicate that PKC is another candidate for rate-limiting molecular switch in hypertrophic signalling. PKC is a family of serine/threonine specific protein kinases and considered as "micro-chip" in the
cell signalling machinery. Biochemical and molecular cloning analysis has revealed so far the existence of 11 PKC isoforms, exhibiting individual characteristics and distinct patterns of tissue distribution. These isoforms can be divided into three groups: classical or conventional PKC (cPKC; α, βI, βII, γ), new or novel PKC (nPKC; δ, ε, η, θ, μ) and atypical PKC (aPKC; ζ, λ). Though the biological significance of this heterogeneity has not been fully clarified, evidence suggests that the members of this enzyme family are activated in specific intracellular compartments in different ways, depending on various membrane lipid metabolites, and play distinct roles in the control of major cellular functions. PKC isoforms are translocated to different cellular sites on activation, via binding to various receptors for activated C-kinases, called RACKs, a mechanism that most likely confers different isoform functions. As suggested by Nishizuka, whose pioneering work discovered PKC two decades ago, the specific functions of each isoform of the PKC family in cellular regulation are a subject of major scientific interest. Activation of PKC induces phosphorylation of many proteins, including various ion pumps, channels and exchangers, in cells causing alterations to a number of biological systems thought to underlie several pathological states. Therefore, the prospects of pharmacological manipulation, directed selectively against PKC, appear quite promising.

A role for PKC in cardiac hypertrophic signalling emerged first from culture models of myocyte hypertrophy. Most or all hypertrophic stimuli directly activate one or more phospholipases, such as phospholipase C, with liberation of diacylglycerol (DAG) and subsequent activation of PKC, typically measured as translocation of PKC activity or immunoreactivity from one cellular compartment to another. PKC is reported to be a key molecule in the myocyte proliferation which occurs in response to mechanical loading and which underlies cardiac hypertrophy. In some instances, there was an increase in tissue-associated PKC in myocytes subjected to “stretching” which induces expression of specific genes associated with proliferation (e.g. c-fos) and this was blocked by H7, a PKC inhibitor. Therefore, it has been generalised that PKC activation causes myocyte hypertrophy and PKC inhibitors can antagonise hypertrophic responses. However, overexpression approaches have critically pointed out that β-PKC isoform is participating in cardiac hypertrophy. Cultured cardiac myocytes overexpressed with β2-PKC exhibited robust induction of the promoters of β-MHC and skeletal α-actin, two marker genes for transcriptional signalling in hypertrophy. Transgenic animal experiments in which β-PKC was overexpressed specifically in cardiac myocytes with α-MHC promoter had resulted in a pathological hypertrophy phenotype. The increase in β-PKC in hypertrophied human hearts and myocytes was also verified in a recent study by Bowling et al. Using left ventricular free wall samples from failing hearts, they showed by immunoblot that two Ca2+-sensitive PKC isoforms, α and β (both β1 and β2 forms), were substantially elevated, by 40 to 70 percent, in particulate or membrane fractions. Further, the α- and β-PKC were increased in cardiac myocytes by immunostaining in tissue sections, and β1 and β2-PKC mRNAs were also higher in failing myocytes in situ hybridisation. PKC enzyme activity in failing membranes in vitro was elevated markedly (>3-fold) and was reduced significantly by an inhibitor of β-PKC, LY335351. Overall, the results support a model of enhanced transcription of β-PKC gene in failing human cardiac myocytes, with consequent elevation in intact β-PKC proteins. Numerous evidences suggest that β-PKC inhibitor, LY335351, might have a therapeutic benefit and this drug has already been known to improve vascular abnormalities in diabetic rats.

Hypertrophy is also seen in mice transgenic for upstream signalling molecules in the PKC pathway. Cardiac hypertrophy and dilatation can result from stimulation of signal transduction pathways mediated by heteromeric G proteins, especially Gq, whose alpha subunit activates phospholipase Cβ. Transgenic mice expressing a hemagglutinin epitope-tagged, constitutively active mutant of the Gq alpha subunit (Haalpα-q) in hearts developed cardiac hypertrophy. Because Haalpα1q acts upstream of calcineurin, it was hypothesised that Haalpα-q might initiate additional pathways leading to hypertrophy and dilatation. Treating Haalpα-q mice with CsA diminished some aspects of hypertrophic phenotype, suggesting that multiple pathways are involved. In another experiment, overexpression of a peptide that blocks Gq signalling antagonises hypertrophy in response to the stimulus of pressure overload. Thus, the culture and transgenic studies taken together suggest multiple pathway for hypertrophic signalling. A pathway could regulate its own activity via autoinduction or complex with other cell signalling systems and result in maintenance of hypertrophic signalling over long times.

**Rationale for Future Work**

The Ca2+/calcineurin studies have opened up several new questions: (i) whether blocking the calcineurin
pathway merely halts or actually reverses the hypertrophy, (ii) can CsA reverse cardiac hypertrophy and heart failure in the transgenic mice after cardiac dysfunction is well established, (iii) how cardiac myocytes distinguish between elevated [Ca\(^{2+}\)]i levels induced by hypertrophic stimuli and that associated with the contraction-relaxation cycles of muscle, (iv) what are the cellular mechanisms that lead to different expression patterns of Ca\(^{2+}\)-transporting proteins, (v) what is the functional significance of tissue-specific NFAT proteins in the heart? (vi) what are the key target genes of NFAT3? (vii) whether CsA or FK506 form a novel therapy for certain forms of human heart failure (despite their adverse side effects)?

On the other hand, therapeutic potential of PKC inhibitors for cardiac hypertrophy is exciting news and is another excellent example of basic cardiac research progressing to drug development. However, the ubiquity of PKC needs to be kept in mind in interpretation of the effects of drugs in the intact animal, and it is conceivable that PKC inhibition could have beneficial effects in one cell type and adverse effects in another. Myocyte culture models should be used to work out the molecular details of hypertrophic signalling, i.e. how exactly various receptors and switches such as β-PKC regulate the myocyte phenotype. Consideration of cell-specific drug effects is important and further work might uncover even better drug targets.

The studies summarised here are no doubt a big step towards uncovering the underlying pathology of cardiac hypertrophy. They also provide a clear cut picture of transcriptional mechanisms underlying cardiovascular development. The successful treatment of hypertrophic animal models with CsA and FK506 suggests a novel therapy for certain forms of human heart disease. However, there is an extensive clinical literature describing adverse side effects associated with long-term CsA therapy, hence new calcineurin inhibitors may need to be developed. Cardiac transplant patients who receive CsA therapy, for example, suffer from nephrogenic toxicity and hypertension. Clinical trials performed to date have not conclusively examined a correlation between CsA and a benefit to patients with various forms of heart disease. Because GATA4 is a heart-specific gene regulator, screening for small molecules that block calcium/calciunmuni/NFAT3/GATA4 interactions might lead to the development of heart-specific drugs. Research on mechanisms of transcriptional signalling should be of special importance, because transcriptional drugs might target myocytes selectively. Any study that provides fundamental insights into the biology of cardiac hypertrophy is the need of the day.

References
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